AD_____

Award Number: DAMD17-02-1-0007

TITLE: Methylselenium and Prostate Cancer Apoptosis

PRINCIPAL INVESTIGATOR: Junxuan Lu, Ph.D.

CONTRACTING ORGANIZATION: The University of Minnesota
Minneapolis, Minnesota 55455-2070

REPORT DATE: February 2005

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	February 2005	Annual (4 Jan 2004 - 3 Jan 2005)

4. TITLE AND SUBTITLE
Methylselenium and Prostate Cancer Apoptosis

5. FUNDING NUMBERS
DAMD17-02-1-0007

6. AUTHOR(S)
Junxuan Lu, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The University of Minnesota
Minneapolis, Minnesota 55455-2070

E-Mail: jlu@hi.umn.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

The purpose of this research is to gain a better understanding of the biochemical pathways and molecular targets for the selective induction of apoptosis signaling and execution of prostate cancer (PCa) cells by methyl selenium (Se)/selenol. We hypothesized that methyl selenium inhibits PI_3K-AKT survival pathway leading to the activation of caspase-dependent apoptosis execution in PCa cells. The specific aims are to delineate the caspase-mediated execution pathways of apoptosis (Objective 1) and to critically test the role of PI_3K-AKT survival pathway in apoptosis signaling (Objective 2) induced by methyl Se/selenol. We have performed experiments pertinent to these two objectives. Specifically, we established an inverse correlation between AKT activity and the sensitivity to MSeA in 3 PCa cell lines; characterized the effects of PI3K and AKT inhibitors as well as MEK-ERK inhibitor on MSeA –induced apoptosis; established the caspase-dependence for apoptosis execution in LNCaP cells induced by MSeA when combined with inhibitors; and showed greater impact on mitochondria cytochrome c release by PI3K-AKT pathway than ERK; established stable activated AKT transfectant DU145 clones and showed a AKT-dose-dependent increase in resistance to MSeA. Finally we also established the specificity of PI3K/AKT and ERK regulation of MSeA induced death was not observed in selenite-induced death in LNCaP cells. These results have been published in Carcinogenesis, 2005 (reprint).

14. SUBJECT TERMS
Selenium, methylselenol, prostate cancer chemoprevention, apoptosis

15. NUMBER OF PAGES
15

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Cover.....1

SF 298.....2

Table of content..... .. 3

Body of Report

Introduction.....4

Detailed report (body) 4

Key Research Accomplishments (bulleted summary) 6

Reportable Outcomes.....6

Conclusions.....6

Appendix 7

Body of Report

1. Introduction

The purpose of this research is to gain a better understanding of the biochemical pathways and molecular targets for the selective induction of apoptosis signaling and execution of PCa cells by methyl Se/selenol. We hypothesized that methyl Se/selenol inhibits PI_3K-AKT survival pathway leading to the activation of caspase-dependent apoptosis execution in PCa cells. The specific aims are to delineate the caspase-mediated execution pathways of apoptosis (Objective 1) and to critically test the role of PI_3K-AKT survival pathway in apoptosis signaling (Objective 2) induced by methyl Se/selenol.

The following is the revised SOW due to change of institutional affiliation and delay in transferring of award to new institution. Active support came through in May 2004)

STATEMENT OF WORK

Objective 1: To define the generalizability and specific features of the caspase-mediated apoptosis execution pathway(s) triggered by methyl Se/selenol pool in PCa cell lines of different androgen dependence and malignancy states.

Test essentiality of caspases and cell detachment for apoptosis induction by methyl Se/selenol in LNCaP, PC-3 and PrEC cells. Correlate PTEN and AKT status with apoptosis sensitivity to methyl Se/selenol.

Delineate caspase cascades invoked by methyl Se/selenol with caspase inhibitors. Test role of mitochondria for apoptosis signaling induced by methyl Se/selenol.

Objective 2: To critically test the mediator role of AKT inhibition as an upstream signaling pathway for methyl Se/selenol-induced PCa apoptosis

Test effects of PI3K inhibitors LY294001 and wortmannin alone or in combination with methyl Se/selenol on apoptosis signaling and execution in DU145 and other PCA cells.

Restore AKT activity in stable transfectants of a constitutively-active AKT (AKT) in DU145 cells. Select for 3 clones expressing low, moderate and high level of the transgene.*

Compare their apoptosis responses to methyl Se/selenol with vector transfectants (control) in dose response and time course experiments.

2. Body of report

We have in the current reporting period conducted experiments related to both objectives above. These are summarized below. The data have been published in ***Carcinogenesis*** 2005 (See reprint, **Appendix 1**).

2.1 Pertinent to Objective 1: We compared and established that the apoptosis sensitivity of DU145, PC-3 and LNCaP prostate cancer cells under regular growing condition (10% serum supplemented complete media) to methylseleninic acid (precursor to methylselelenol) was inversely correlated to their AKT activity. In our hands, the phospho-AKT (indicative of active form) of the 3 cell lines showed the following ascending order: DU145 <LNCaP <PC-3. The apoptosis sensitivity to methylseleninic acid (MSeA) followed the inverse order: DU145 (low dose required) > LNCaP > PC-3 (high dose required) (**Figure 1** in Reprint).

2.2 Pertinent to Objectives 1 and 2: We characterized caspase-activation patterns induced by MSeA in LNCaP cells, especially under conditions where inhibitors of PI3K/AKT and/or ERK pathways were used (**Figure 3** in Reprint). We showed that both caspase-8 and caspase-9 pathways were activated and cytochrome c release from mitochondria was involved. We established that apoptosis induced under these conditions are caspase-dependent because the general caspase inhibitor zVADfmk completely blocked apoptosis (**Figure 4,** reprint). Although both caspase-8 and 9 pathways were activated, we showed that the caspase-8 (suggestive of death receptor-extrinsic pathway) inhibitor was more protective than caspase-9 (mitochondria cytochrome c-intrinsic pathway) inhibitor under condition of PI3K and/or ERK inhibition (**Figure 4**).

2.3 Pertinent to Objective 2: We compared AKT inhibitors with PI3K inhibitor LY294002 for enhancing apoptosis induced by MSeA in LNCaP cells, and found that specific AKT inhibition greatly synergized with MSeA on apoptosis induction (**Figure 5, reprint**). This experiment provided further specificity of the molecular regulator of apoptosis in the PI3K pathway for the sensitivity towards MSeA in LNCaP cells.

2.4 Pertinent to Objective 2: We established and characterized several clones of stable transfectants of activated AKT in DU145 cells and compared their sensitivity to undergo apoptosis after exposure to MSeA. As shown in **Figure 6** in reprint (2 clones reported), transfection of DU145 cells with a constitutively active AKT rendered them more resistant than the vector-transfectant to MSeA in AKT dose-dependent manner.

2.5 Pertinent to both Objectives: We showed that caspase-mediated apoptosis execution induced by a different selenium-sodium selenite in LNCaP cells was not affected by inhibitors of PI3K or ERK pathway (**Figure 2**, reprint).

Together, the data generated so far support the hypothesis that PI3K-AKT pathway activity plays a determinant role for the apoptosis sensitivity of PCa cells to MSeA, which can be further modulated by a treatment-induced activation of ERK1/2 in LNCaP cells. AKT and ERK1/2 differentially modulated cytochrome c involvement in MSeA-induced extrinsic (major) and intrinsic caspase cascades in the LNCaP cells. These pathways appeared to play minor if not negligible role in selenite-induced caspase-mediated apoptosis in LNCaP cells.

3. Key accomplishments

- We established an inverse correlation between AKT activity and sensitivity to MSeA in 3 PCa cell lines
- We characterized the effects of PI3K and AKT inhibitors as well as MEK-ERK inhibitor on MSeA –induced apoptosis in LNCaP cells.
- We established the caspase-dependence for apoptosis execution in LNCaP cells induced by MSeA when combined with inhibitors and showed greater impact on mitochondria cytochrome c release by PI3K-AKT pathway than ERK.
- We established stable activated AKT transfectant DU145 clones and showed a AKT-dose-dependent increase in resistance to MSeA
- We established specificity of PI3K/AKT and ERK regulation of MSeA induced death was not observed in selenite-induced death in LNCaP cells

4. Reportable outcomes

4.1 Peer-reviewed publication

Hongbo Hu, Cheng Jiang, Guangxun Li and Junxuan Lü: PKB/AKT and ERK regulation of caspase-mediated apoptosis by methylseleninic acid in LNCaP prostate cancer cells. *Carcinogenesis* 2005 26(8):1374-1381. Advance Access originally published online on April 21, 2005.

4.2 Grant award

Source:	National Cancer Institute
Number:	1R01CA95642
Title:	Selenium and prostate cancer apoptosis pathways
Principal investigator:	Lu, Junxuan
Dates of entire period:	September. 2004-August 2008

The goal of this grant is to investigate the biochemical and molecular pathways through which selenite and other selenium compounds induce prostate cancer cell apoptosis. The role of ATM-p53 is a key emphasis.

5. Conclusions

Work conducted during this current reporting period has further strengthened the specific role of PI3K-AKT pathway in modulating methyl Se induced caspase-mediated death, leading to the inference that the AKT (survival protein kinase) may be a key determinant of chemopreventive and therapeutic efficacy of methyl Se. In the next funding period, we would like to define the early signaling mechanisms induced by

methylselenium for apoptosis and how and through what targets AKT regulates death signaling. In addition, we will initiate work with PrEC cells to compare and contrast how "normal" prostate epithelial cells respond to MSeA and selenite.

6. Appendix-reprint

Hongbo Hu, Cheng Jiang, Guangxun Li and Junxuan Lü: PKB/AKT and ERK regulation of caspase-mediated apoptosis by methylseleninic acid in LNCaP prostate cancer cells. *Carcinogenesis* 2005 26(8):1374-1381. Advance Access originally published online on April 21, 2005.

Carcinogenesis vol.26 no.8 pp.1374–1381, 2005
doi:10.1093/carcin/bgi094
Advance Access publication April 21, 2005

PKB/AKT and ERK regulation of caspase-mediated apoptosis by methylseleninic acid in LNCaP prostate cancer cells

Hongbo Hu, Cheng Jiang, Guangxun Li and Junxuan Lü*

The Hormel Institute, University of Minnesota, 801 16th Avenue NE, Austin, MN 55912, USA

*To whom correspondence should be addressed. Tel: +1 507 437 9680; Fax: +1 507 437 9606;
Email: jlu@hi.umn.edu

Methylselenol has been implicated as an active metabolite for the anticancer effect of selenium in part through the induction of cancer cell apoptosis. Since inactivation of the AKT/protein kinase B negative regulator gene PTEN (phosphatase and tensin homologue deleted on chromosome 10) is common in prostate cancer (PCa), we compared PTEN wild-type DU145 PCa cells (low basal AKT activity) with PTEN-mutant LNCaP PCa cells (high basal AKT activity) for their apoptosis responses to the methylselenol precursor methylseleninic acid (MSeA) and sodium selenite, an inorganic salt. Our results show that LNCaP cells withstood ~4 times higher doses of MSeA than DU145 cells, although they were slightly more sensitive than the latter to selenite-induced apoptosis. Treatment by MSeA modestly attenuated AKT phosphorylation and increased phospho-ERK1/2 in LNCaP cells. Selenite treatment increased the phosphorylation of p53 Ser15 and both kinases, but the selenite-induced apoptosis was not influenced by chemical inhibitors of either kinase. In contrast, PI3K/AKT inhibitors greatly sensitized LNCaP cells to apoptosis induced by MSeA, accompanied by increased mitochondrial release of cytochrome *c* and multiple caspase activation without changing p53 Ser15 phosphorylation. The apoptosis was further accentuated by extracellular signal regulated kinases 1 and 2 (ERK1/2) inhibition without further increase in cytochrome *c* release. The general caspase inhibitor z-VAD-fmk completely blocked MSeA-induced apoptosis when both kinases were inhibited, whereas a caspase-8 inhibitor exerted a greater protection than did a caspase-9 inhibitor. Transfection of DU145 cells with a constitutively active AKT increased their resistance to MSeA-induced apoptosis. In summary, AKT played an important role in regulating apoptosis sensitivity of LNCaP and DU145 cells to MSeA. An MSeA-induced activation of ERK1/2 in LNCaP cells also contributed to resistance to apoptosis. However, these kinases did not significantly regulate caspase-mediated apoptosis induced by selenite in LNCaP cells. These findings support the differential involvement of these protein kinase pathways in regulating apoptosis induction by different forms of selenium.

Abbreviations: ERK, extracellular signal regulated kinase; GSK-3, glycogen synthase kinase-3; MSeA, methylseleninic acid; PCa, prostate cancer; PI3K, phosphatidylinositol 3-kinase; PARP, poly(ADP-ribose) polymerase; PTEN, phosphatase and tensin homologue deleted on chromosome 10.

Introduction

Despite advances in early detection and treatments, prostate cancer (PCa) continues to be one of the biggest health problems for aging men in USA. Almost 200 000 new cases were projected in 2004 and 30 000 men will succumb to this disease (1) (http://www.cancer.org/docroot/stt/stt_0.asp). Chemoprevention using synthetic or naturally occurring agents that inhibit one or more steps in the natural history of prostate carcinogenesis holds great promise to decrease the morbidity and mortality of PCa (2). The essential trace element selenium has been shown as a promising preventive agent for PCa in a human clinical trial by Clark and co-workers (3,4) and additional trials are being conducted to verify this efficacy (5–7). Mechanistically, induction of apoptosis is believed to be a critical cellular event in PCa chemoprevention and therapy by selenium compounds (8). Methylselenol has been implicated as an active anticancer selenium metabolite (9–13).

We have earlier shown that methylselenol and its synthetic penultimate precursor compound methylseleninic acid (MSeA) induce caspase-mediated apoptosis in DU145 PCa cells (14,15) and have observed that cell death is associated with decreased phosphorylation of AKT/protein kinase B and extracellular signal regulated kinases 1 and 2 (ERK1/2) (14–16). The phosphatidylinositol 3-kinase (PI3K)–AKT pathway has been shown to inhibit apoptosis in most cell types (17,18) and promote angiogenesis (19). In ~50% of prostate tumors, this pathway is constitutively upregulated owing to the deletion of the tumor suppressor PTEN (phosphatase and tensin homologue deleted on chromosome 10) (20–22), which functions as a negative regulator of PI3K in part through lipid phosphatase activity (23). The ERK1/2 pathway is primarily known for mitogenic signaling and for modulating cell survival in most model systems (24,25). Constitutive activation of ERK1/2 has been observed in some cancer types including the DU145 PCa cells (26,27). These findings support the important roles of the PI3K/AKT and ERK1/2 signaling pathways in PCa development. It is of significant interest in terms of targeted therapy or chemoprevention whether the mutational/functional status of these pathways could be molecular regulators or determinants of the potency of selenium for apoptosis induction in PCa cells.

To this end, we have compared the sensitivity of three classic PCa cell lines for the induction of apoptosis by MSeA and selenite, an inorganic salt that we and others have shown to induce DNA single strand breaks (28–30). The androgen-independent DU145 cells possess a wild-type PTEN and low basal AKT activity, and constitutively active ERK1/2 (26,27). The androgen-sensitive LNCaP cells and androgen-independent PC-3 cells are PTEN negative with greater basal AKT activity than the DU145 cells (26,27). We report here that LNCaP and PC-3 cells are more resistant to the induction of apoptosis by MSeA than DU145 cells in a manner proportional to their basal AKT activities. We show that inhibition of AKT

by either a PI3K inhibitor or AKT inhibitors sensitized LNCaP cells to the induction of apoptosis by MSeA, whereas transfection of a constitutively active AKT (CA-AKT) into DU145 cells increased their resistance to MSeA. Furthermore, inhibiting MSeA-induced ERK1/2 activation accentuated apoptosis in LNCaP cells, implicating a unique survival response that further contributed to the resistance phenotype. However, inhibiting these two pathways did not affect selenite-induced apoptosis in LNCaP cells.

As far as caspase mediators are concerned, we focused on caspase-8 and caspase-9 as representatives of two well characterized caspase activation pathways (31,32). The mitochondrial (intrinsic) pathway generally involves the loss of transmembrane potential and the release of cytochrome *c* into the cytosol. Cytosolic cytochrome *c* associates with APAF-1 and procaspase-9 to form a protein complex known as the apoptosome, leading to the cleavage activation of procaspase-9, which in turn cleaves and activates the effectors caspase-3 and caspase-7, and finally to the characteristic proteolysis, e.g. poly(ADP-ribose) polymerase (PARP) cleavage (33) and DNA digestion. The death receptor (extrinsic) pathway involves the engagement of the death receptors, recruits the adapter protein FADD and procaspase-8 to form a complex known as the death inducing signaling complex or DISC. The consequent proximity of procaspase-8 proteins in the DISC allows their autocleavage activation. Caspase-8 can directly activate caspase-3, and caspase-7 to lead to PARP cleavage and DNA oligonucleosomal digestion. Caspase-8 can also activate caspase-9 cascade by cross-talk through cleaving Bid, leading to the translocation of the truncated Bid to the mitochondria where it facilitates the release of cytochrome *c* and the activation of the intrinsic pathway. Our data support AKT and ERK suppression of both extrinsic and intrinsic caspase cascades to attenuate apoptosis sensitivity for MSeA in LNCaP cells without affecting p53 phosphorylation.

Materials and methods

Chemicals and reagents

MSeA (CH_3SeO_2H) was synthesized as a precursor for methylselenol for cell culture studies (11,12) and was a generous gift of Dr Howard Ganther, University of Wisconsin. We have shown earlier that methylselenol generated by reacting selenomethionine with recombinant methioninase recapitulated morphological apoptosis and biochemical markers induced by MSeA (15). PI3K inhibitor LY294002, AKT specific inhibitor (Catalog No. 124005) and NL-71-101 (Catalog No. 487940) were purchased from CalBiochem (La Jolla, CA). MEK inhibitor U0126 was purchased from Promega (Madison, WI). Caspase inhibitors (z-VAD-fmk, z-IETD-fmk and z-LEHD-fmk) were purchased from MP-Biomedical (Aurora, OH). Phospho-specific antibodies for AKT (Ser473), ERK1/2 (Thr202/Tyr204) and caspase antibodies used in this study were purchased from Cell Signaling Technology (Beverly, MA). Mitochondria-free cytosol fractionation kit and cytochrome *c* antibody were purchased from BD Biosciences (Palo Alto, CA).

Cell culture and treatments

DU145, LNCaP and PC-3 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). LNCaP cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine without antibiotics. DU145 cells were cultured in Minimum Essential Eagle's medium supplemented with 10% fetal bovine serum and 2 mM L-glutamine without antibiotics. PC-3 cells were cultured in F-12K medium supplemented with 10% FBS and 2 mM L-glutamine without antibiotics. Cells at 50–60% confluence, usually 24–48 h after plating, were given a medium change and treated with selenium. PI3K, AKT or MEK inhibitors were dissolved in dimethylsulfoxide (DMSO) as concentrated stocks. In experiments involving the kinase inhibitors, fresh media containing a desired concentration of each inhibitor were fed to cells for 1 h and then additional treatments were initiated. Caspase inhibitors and MSeA were mixed into treatment media first and then fed to cells. DMSO (2 μl/ml or less) was added to groups that did not receive the inhibitors to control the solvent vehicle effects. DMSO at the concentration used did not by itself cause any observable adverse morphological responses.

Apoptosis and caspase assays

After treatments, both adherent and floating cells were collected. A cell death ELISA kit (Roche Diagnostics Corporation, Indianapolis, IN) was used to measure oligonucleosomes released by apoptotic cells. Cleavages of PARP and procaspases were detected by immunoblot as markers of caspase-mediated apoptosis as described previously (14). Caspase activity was measured as described previously with assay kits from R&D Systems (Minneapolis, MN) (34).

Preparation of cytosolic extracts

A Cell Fractionation Kit (Clontech-BD Biosciences, Palo Alto, CA) was used to prepare cytosolic extracts for cytochrome *c* detection. Both adherent and floating cells were collected by centrifugation. The cells were resuspended in 1 ml of ice-cold wash buffer provided by the Kit. After washing, the cell pellets were resuspended in 0.8 ml of ice-cold fractionation buffer and incubated on ice for 10 min. The cells were homogenized in an ice-cold Dounce tissue grinder with 60 passes. After centrifugation at 12 000 *g* for 25 min, the mitochondria-free supernatant was collected for immunoblot analysis of cytochrome *c*.

Stable transfection of DU145 cells with a CA-AKT1

The mouse CA-AKT1 in pUSEamp plasmid was kindly provided by Dr Zigang Dong (Hormel Institute, University of Minnesota). DU145 cells were seeded into 6-well plates until ~60% confluence and were transfected using Lipofectamine 2000 purchased from Invitrogen (Carlsbad, CA). Briefly, 2 μg of plasmid and 10 μl of Lipofectamine 2000 were diluted into 100 μl of serum-free medium, respectively. After 5 min incubation at room temperature, the two solutions were combined with gentle mixing. After incubation for 20 min at room temperature, the mixture was added to each well which contained 0.8 ml serum free medium. After 6 h incubation at 37°C, the transfection medium was replaced with 2 ml of complete medium. After 24 h incubation, the transfected cells were harvested and replated into 100 mm plates and replaced with selection medium containing 800 μg/ml of G418 the following day. Resistant colonies were selected after ~6 weeks. The AKT activity of each clone was measured as described below.

In vitro AKT kinase assay

In vitro AKT kinase assay was carried out as per the manufacturer's instructions by an AKT Kinase Assay Kit purchased from Cell Signaling Technology (Beverly, MA). Briefly, the cells were harvested and washed twice with phosphate buffered saline, and lyzed in ice-cold lysis buffer provided by the kit. Then 200 μg of protein was immunoprecipitated with 2 μg of anti-AKT antibody overnight. After extensive washing, the immunoprecipitates were incubated with 1 μg of glycogen synthase kinase-3 (GSK-3) fusion protein substrate in 50 μl of kinase buffer for 30 min at 30°C. Reactions were terminated by SDS loading buffer. The samples were separated on 12% SDS–PAGE, and the phospho-GSK-3α/β (Ser219) was detected by immunoblotting.

Statistical analysis

Where appropriate, ANOVA was used to test the significance of the differences among the treatment groups and their relative controls. Statistical significance was determined at the 0.05 or 0.01 level using Student's *t*-test.

Results

LNCaP and PC-3 cells were less sensitive than DU145 cells to MSeA-induced apoptosis

We first validated the status of AKT and ERK1/2 in the three cell lines during exponential growth phase by western blot analysis (Figure 1A). As expected, LNCaP cells expressed androgen receptor (AR) and prostate specific antigen (PSA), whereas the two-androgen independent cell lines did not express either molecule. AKT (Ser473) phosphorylation was detected in the following order in the three cell lines: DU145 < LNCaP < PC-3. However, much higher levels of phospho-ERK1/2 were detected in DU145 cells than in LNCaP or PC-3 cells.

To compare their apoptotic sensitivity to MSeA, the cells were treated with increasing concentrations of MSeA for 24 h.

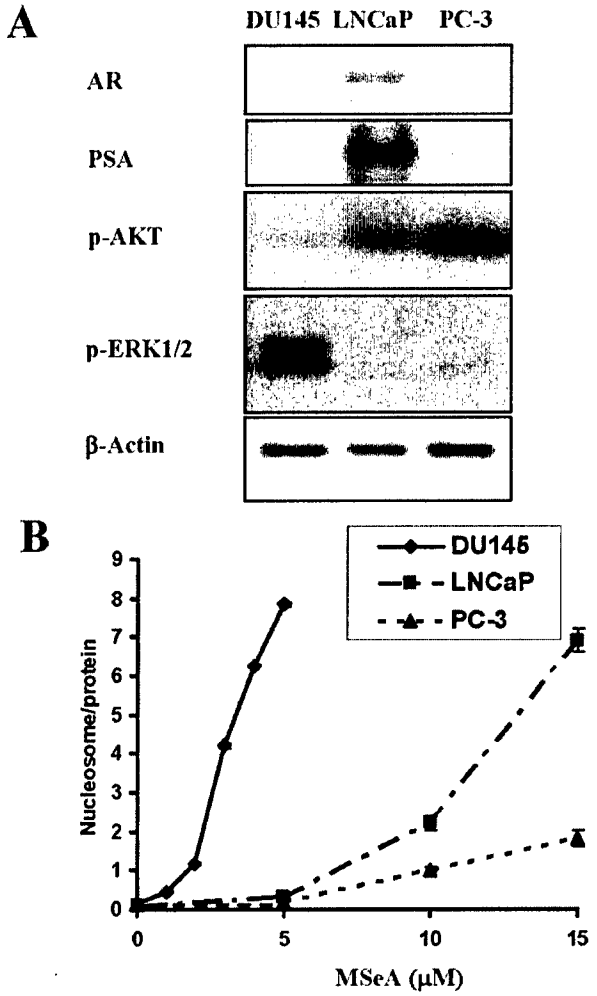

Fig. 1. (**A**) Immunoblot detection of AR, PSA and phospho-AKT and phospho-ERK1/2 in DU145, LNCaP and PC-3 cells lysate. (**B**) ELISA detection of apoptotic oligonucleosomes in DU145, LNCaP and PC-3 cells after 24 h treatment with increasing concentrations of MSeA. Floating and adherent cells were collected for apoptosis assay with cell death ELISA Kit. ELISA OD was normalized to protein content. Each point represents mean and SD of 4 replicates.

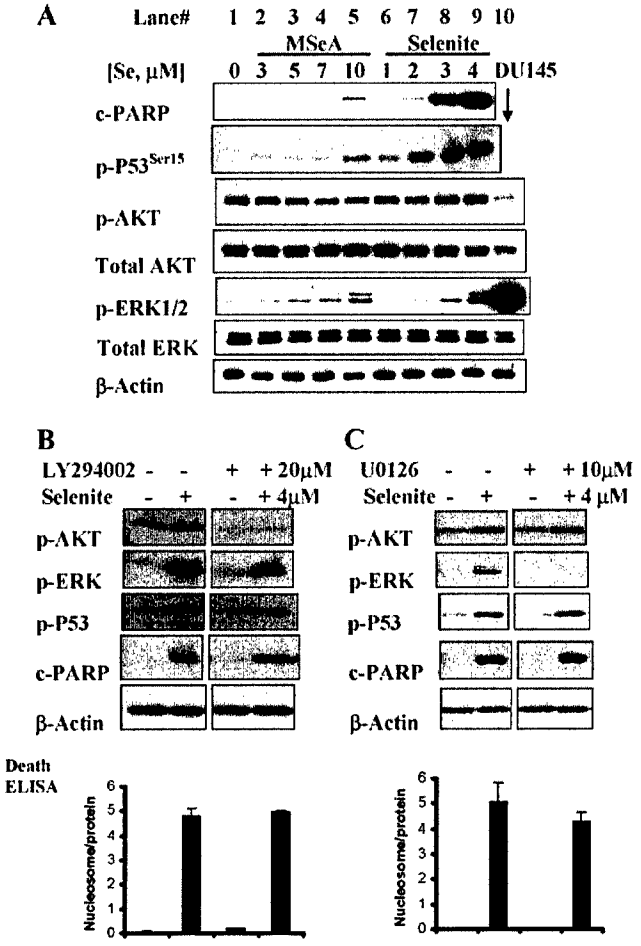

Fig. 2. (**A**) Immunoblot analyses of cleaved PARP (89 kDa) and the phosphorylation status of AKT and ERK1/2 and p53 in LNCaP cells after 24 h exposure to MSeA (lanes 2–5) and selenite (lanes 6–9). Lane 10 was DU145 cell lysate for comparison. β-Actin expression was probed to indicate the evenness of loading of the protein extract from each treatment. (**B**) Effect of PI3K inhibitor LY294002 and (**C**) effect of MEK inhibitor U0126 on caspase-mediated cleavage of PARP and overall death induced by selenite after 24 h exposure. Cells were pretreated with LY294002 or U0126 for 1 h before the addition of selenite for 24 h (total inhibitor exposure time was 25 h).

Apoptosis was estimated by Cell Death ELISA Kit. As shown in Figure 1B, a significant increase of apoptosis was observed in DU145 cells in response to MSeA exposure between 2 and 3 μM. To elicit the same death response in LNCaP cells and PC-3 cells 4 and 7 times more MSeA were required, respectively. The apoptosis responses of the three cell lines showed an inverse association with their basal AKT phosphorylation levels.

LNCaP cells retained sensitivity to caspase-mediated apoptosis by selenite

To examine whether LNCaP cells are resistant to apoptosis induced by other forms of selenium, we compared the death response induced by MSeA with sodium selenite, which has been known to induce DNA damage and genotoxicity in several cell types (28–30). As shown in Figure 2A, while the caspase-mediated PARP cleavage was detected at a low level in LNCaP cells exposed to 10 μM of MSeA (lane 5), 3 μM of selenite was sufficient to induce significant PARP cleavage (lane 8). As a reference value, 5 μM of selenite was needed to induce apoptotic DNA laddering in the absence of activation of caspases in DU145 cells (14). These results suggest that LNCaP cells possess mechanisms that suppress the MSeA-induction of caspase activation and apoptosis, and these mechanisms did not apply to the caspase activation pathways induced by selenite.

Effects of MSeA and selenite on phosphorylation status of AKT and ERK1/2

To probe the involvement of AKT and ERK1/2 pathways in regulating apoptosis induced by selenium in LNCaP cells, we assessed the effects of MSeA and selenite on the level of phospho-AKT and ERK1/2 after 24 h treatment (Figure 2A). MSeA exposure resulted in a dose-dependent yet modest decrease of AKT phosphorylation (lanes 2–5). Even with the highest dose of MSeA (lane 5), the remaining level of phospho-AKT was still much higher than in DU145 cells (lane 10). Contrary to the expectation based on our results in

DU145 cells (14–16), MSeA treatment increased ERK1/2 phosphorylation in LNCaP cells in a dose-dependent manner (lanes 2–5), although the absolute magnitude of this increase was small in comparison with the basal phospho-ERK1/2 in DU145 cells (lane 10). MSeA exerted minimal effect, if any, on p53 Ser15 phosphorylation (lanes 2–5). Selenite exposure at doses that led to significant PARP cleavage increased the phosphorylation of both AKT and ERK1/2 in LNCaP cells (lanes 8–9) and p53 Ser15 phosphorylation (lanes 7–9), as reported previously (34).

To test the significance of AKT and ERK phosphorylation during selenite-induced apoptosis, we examined the impact of PI3K inhibitor LY294002 (Figure 2B) and MEK inhibitor U0126 (Figure 2C) on PARP cleavage and overall death as detected by death ELISA. Whereas each inhibitor decreased the phosphorylation of the intended downstream target kinase, they did not significantly increase caspase-mediated apoptosis as indicated by the similar extent of cleavage of PARP or overall death. The results suggest that AKT and/or ERK1/2 do not play an important role in regulating apoptosis induced by selenite in LNCaP cells.

PI3K and MEK inhibitors sensitized LNCaP cells to MSeA-induced apoptosis

To test whether the sustained AKT activation and/or treatment-induced ERK activity in MSeA-exposed LNCaP cells decreased their sensitivity for apoptosis, we next examined the effects of PI3K inhibitor LY294002 and MEK inhibitor U0126, alone or in combination, on apoptosis induced by MSeA. As shown in Figure 3, LY294002 completely inhibited AKT phosphorylation (Figure 3B, lane 3 versus 1) and resulted in a small increase of apoptosis (Figure 3A, column 3 versus 1). Combining LY294002 with MSeA increased apoptosis ~4 times more than the sum of apoptotic signal induced by each agent alone (Figure 3A, column 4).

Treatment with U0126 abolished MSeA-induced ERK1/2 phosphorylation (Figure 3B, lane 6 versus 2). U0126 alone did not have any effect on background apoptosis (Figure 3A, column 5). Combining U0126 with MSeA increased apoptosis modestly (Figure 3A, column 6 versus 2) and the extent of death was about one-third of that induced by the LY294002/MSeA combination (column 6 versus 4).

When PI3K and MEK were inhibited simultaneously, cell death was comparable with that induced by PI3K inhibition alone (Figure 3A, column 7 versus 3). When the two inhibitors were combined with MSeA, we observed a super-enhancement of apoptosis (Figure 3A, column 8 versus 4 and 6). The enhanced apoptosis execution by either inhibitor or both combined did not involve an increase of p53 Ser15 phosphorylation (Figure 3B). Collectively, the data support sustained AKT activation in LNCaP cells as a key factor for suppressing apoptosis signaling from MSeA. Furthermore, MSeA-induced ERK1/2 activation provided an additional survival response to inhibit apoptosis signaling in LNCaP cells. Their effects did not involve p53 Ser15 phosphorylation.

Involvement of caspase-8 and caspase-9 cascades

To explore the caspase targets through which the PI3K and/or MEK inhibitor sensitized LNCaP cells to undergo MSeA-induced apoptosis, we examined the cleavage and activity of key caspases in both the intrinsic (caspase-9, Figure 3B) and extrinsic (caspase-8, Figure 3C) pathways (28,29). Caspase-3 cleavage patterns (Figure 3B) and activities (data not shown)

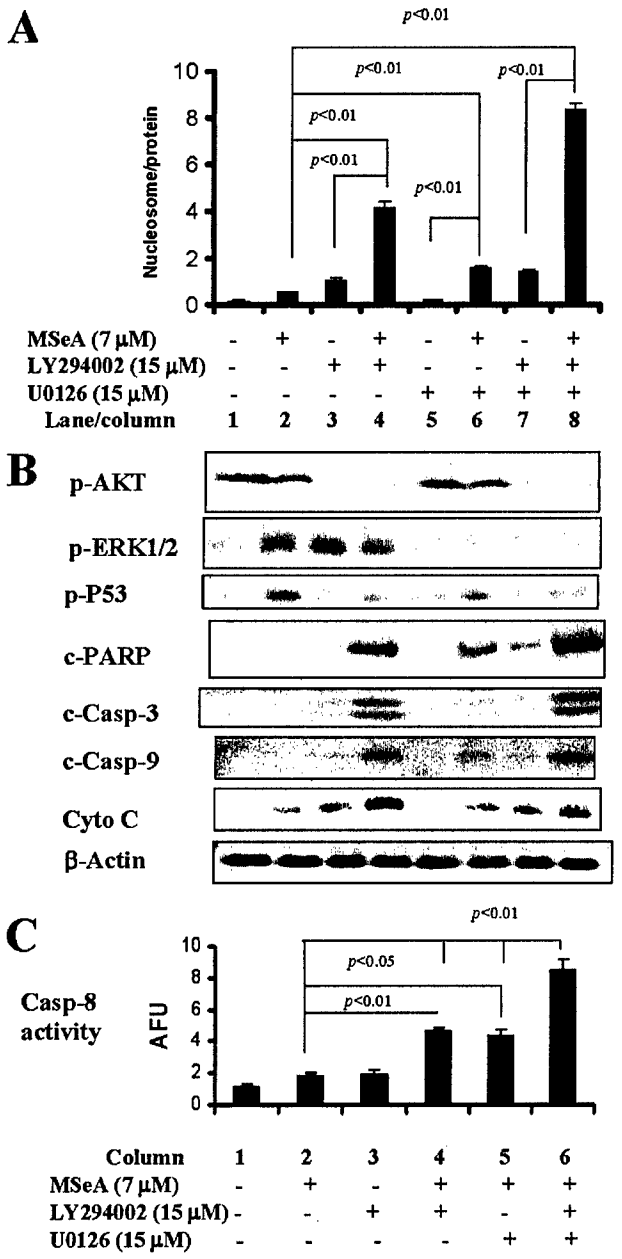

Fig. 3. (**A**) Effects of the PI3K inhibitor LY294002 and/or the MEK inhibitor U0126 on MSeA-induced apoptotic nucleosome release in LNCaP cells detected by ELISA. Cells were pretreated with LY294002 and/or U0126 for 1 h before the addition of MSeA for 24 h (total inhibitor exposure time was 25 h). Each column represents mean and SD of four replicates. (**B**) Immunoblot verification of inhibition of PI3K/AKT and MEK/ERK pathways by chemical inhibitors and detection of phospho-P53 Ser15, cleaved PARP, caspase-3 and caspase-9 and cytosolic cytochrome *c*. Experimental design was identical to (A). β-Actin expression was probed to indicate the evenness of loading of the protein extract from each treatment. (**C**) Caspase-8 activity assay in cells treated with MSeA and/or inhibitors. AFU, arbitrary fluorescence unit. Experimental design was identical to (A) except the omission of U0126 and U0126 plus LY294002 groups. Activity assay for caspase-3 and caspase-9 showed identical patterns as the cleavage patterns shown here in (B) (data not shown).

were in excellent agreement with the extent of PARP cleavage (Figure 3B) and with death ELISA data presented in Figure 3A, consistent with this executioner caspase as an ultimate mediator of PARP cleavage and DNA fragmentation in

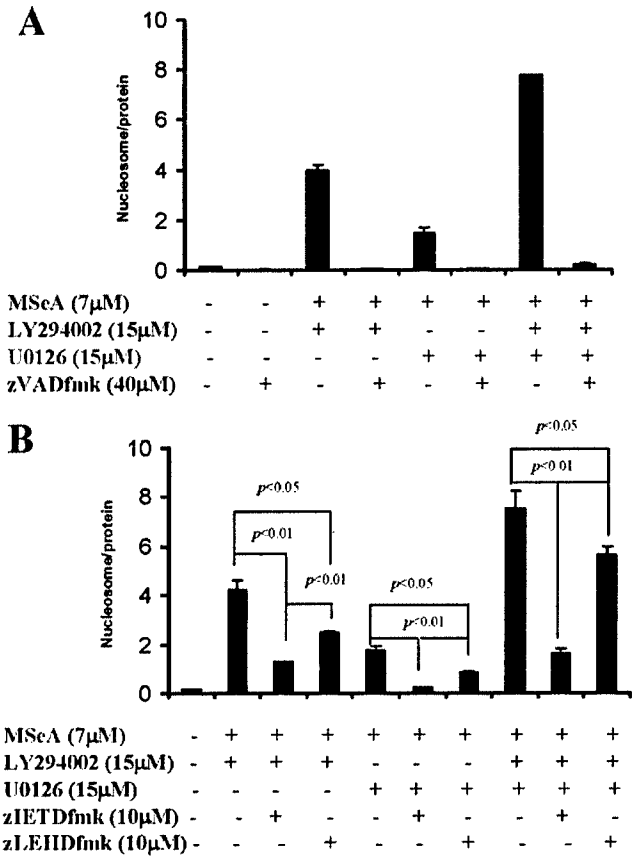

Fig. 4. Effects of (**A**) pan-caspase-inhibitor z-VAD-fmk and (**B**) caspase-8 inhibitor zIETDfmk or caspase-9 inhibitor zLEHDfmk on apoptosis induced by MSeA in combination with PI3K and/or ERK inhibitors in LNCaP cells. Exposure was for 24 h. Floating and adherent cells were combined for the death ELISA. Each column represents mean and SD of four replicates.

MSeA-induced apoptosis. The MSeA/LY294002 combination (Figure 3B, lane 4) and MSeA/ U0126 combination (Figure 3B, lane 6) led to a similar enhancement of caspase-8 activity (Figure 3C, column 4 versus 5), yet their effects on caspase-9 and caspase-3 (Figure 3B, lane 4 versus 6) and apoptosis (Figure 3A, column 4 versus 6) differed by ~3 fold. This difference correlated to a lack of an effect of ERK inhibition on mitochondrial release of cytochrome *c* in MSeA-exposed cells (lane 6 versus 2). In contrast, PI3K/AKT inhibition led to a greater than additive increase in cytochrome *c* release in the MSeA-exposed cells (lane 4 versus 2). These results indicate that both PI3K/AKT and MEK/ERK suppressed caspase-8 activation, yet only the PI3K/AKT pathway exerted a major impact on cytochrome *c* release from mitochondria in MSeA-treated cells. This was further supported by the additive nature of caspase-8 activation when both pathways were inhibited (Figure 3C, column 6) without further enhancing cytochrome *c* release (Figure 3B, lane 8 versus 4).

To verify that the activation of caspases was crucial for apoptosis execution by MSeA in LNCaP cells when PI3K and/or MEK kinases were suppressed, we tested the effect of a general caspase inhibitor zVADfmk (40 μM) on apoptosis. As shown in Figure 4A, zVADfmk completely blocked the apoptosis induced by MSeA in combination with PI3K and/or MEK inhibitor. We next investigated the relative contribution of the two caspase cascades by using specific inhibitors. As shown in Figure 4B, the caspase-8 inhibitor zIETDfmk blocked MSeA/LY-, MSeA/U0126- and MSeA/LY/U0126-induced death by ~70, ~95 and ~80%, respectively. However, the caspase-9 inhibitor zLEHDfmk at the same concentration of 10 μM only exerted ~40, ~50 and ~20% protective effects on the corresponding treatment combinations mentioned above. Taken together, the data indicate that the extrinsic caspase-8 cascade played a greater role than the intrinsic caspase-9 cascade in mediating MSeA-induced apoptosis in LNCaP cells when their PI3K/AKT and MEK/ERK1/2 activities were inhibited and that these two pathways regulated common (caspase-8) as well as distinct (mitochondria/cytochrome *c*) targets in the caspase activation cascades.

Effects of AKT inhibitors on MSeA-induced apoptosis in LNCaP cells

PI3K inhibition by LY294002 could be expected to and did decrease the activity of AKT as shown in Figures 2B and 3B, but could also affect the activities of other PI3K downstream target molecules. In order to establish a specific effect of AKT for regulating the apoptosis sensitivity of LNCaP to MSeA, we tested whether AKT inhibitors could similarly sensitize LNCaP cells to MSeA. NL-71-101, a modified PKA inhibitor that showed greater selective inhibition of AKT than its parental compound (35), exerted an enhancement action nearly identical to that of the PI3K inhibitor LY294002 (Figure 5A). The AKT-specific inhibitor, a 3-(hydroxymethyl)-bearing phosphatidylinositol ether lipid analogue (36), did not cause apoptosis at concentrations as high as 50 μM after 24 h exposure, but greatly sensitized LNCaP cells to MSeA-induced apoptosis in a dose-dependent manner (Figure 5B). Immunoblotting confirmed that this inhibitor significantly decreased the phospho-AKT level and increased caspase-3 cleavage activation and the cleavage of PARP (Figure 5C). These results indicated that AKT inhibition alone was insufficient to induce apoptosis in LNCaP cells within the duration of exposure of ~25 h, but was responsible for sensitizing LNCaP cells to MSeA.

Transfection of DU145 with an active AKT increased their resistance to MSeA

To test the prediction that upregulation of AKT activity in DU145 cells should make them more resistant to MSeA, we established stable transfectants expressing a CA-AKT and examined the effects on MSeA-induced apoptosis. Two clones (clones 8 and 11) expressing different levels of AKT activity (Figure 6A) were compared with vector-transfectants. As expected, the vector-transfectants remained sensitive to MSeA-induced apoptosis, whereas the cells transfected with CA-AKT became more resistant as indicated by fewer round-up cells (Figure 6B) and lower death ELISA readout in inverse proportion to their AKT levels (Figure 6C).

Discussion

Data presented above provided several lines of evidence supporting AKT as a key protein kinase for regulating the apoptosis sensitivity to MSeA in LNCaP and DU145 cells. The association of basal AKT activity with differential sensitivity of the three PCa cell lines (Figure 1) provided the initial clue. The sensitization of LNCaP cells to undergo MSeA-induced apoptosis through attenuating AKT activity with the PI3K inhibitor LY294002 (Figures 3 and 4), with a PKA

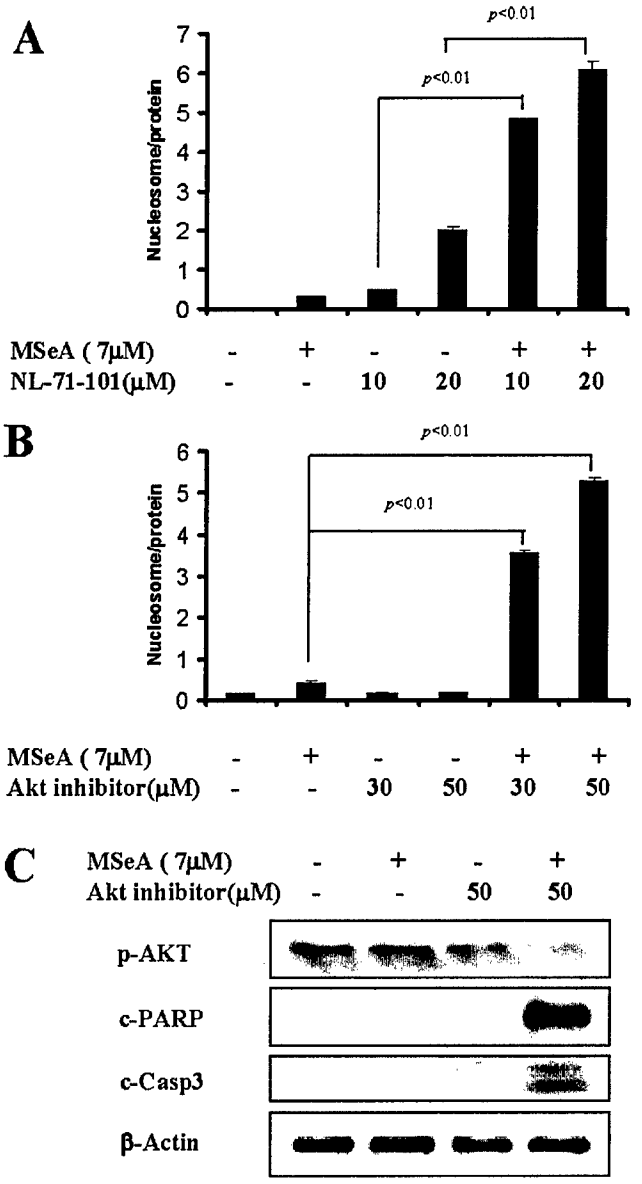

Fig. 5. Effects of AKT inhibitors on apoptosis induced by MSeA in LNCaP cells. (**A**) PKA inhibitor-based AKT inhibitor NL-71-101 (Catalog No. 487940, CalBiochem, La Jolla, CA). (**B**) and (**C**) AKT specific inhibitor [3-(hydroxymethyl)-bearing phosphatidylinositol ether lipid analog, Catalog No. 124005. CalBiochem, La Jolla, CA]. Cells were pretreated with the inhibitors for 1 h before the addition of MSeA for 24 h (total inhibitor exposure time was 25 h). Floating and adherent cells were combined for the death ELISA (**B**) and for immunoblotting analyses (**C**). Each column represents mean and SD of four replicates.

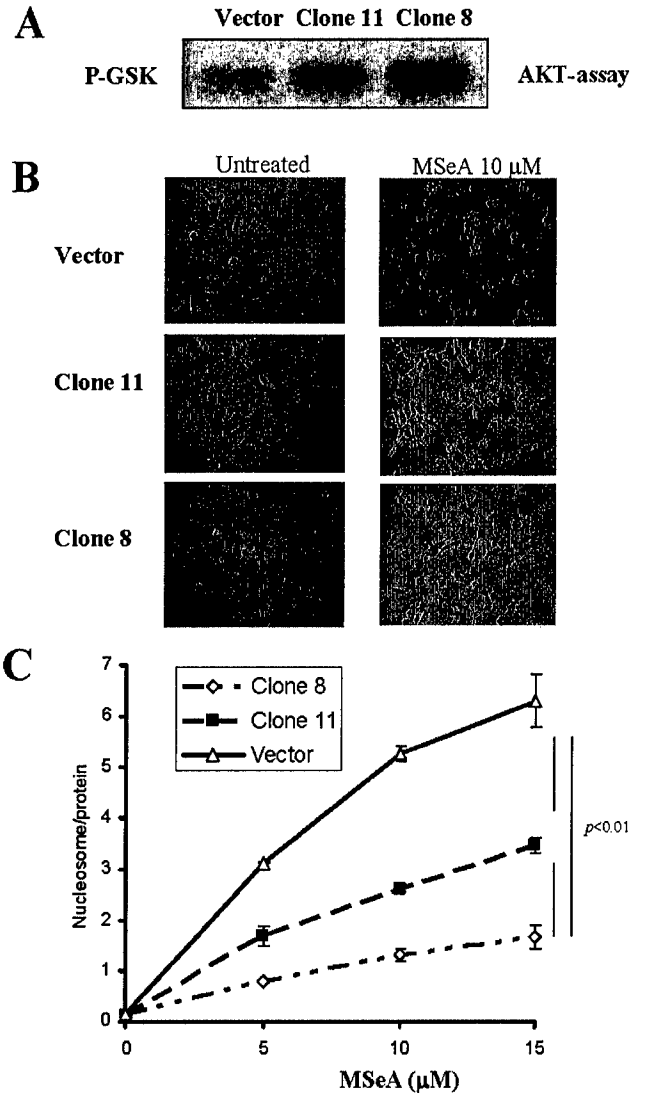

Fig. 6. Effect of expression of a CA-AKT in DU145 cells on MSeA-induced apoptosis. (**A**) AKT activity assay for cell lysate of vector- and AKT-transfectants. Phosphorylation of GSK-fusion protein substrate by immunoprecipitated AKT was detected by immunoblot. (**B**) Phase contrast photomicrograph of morphological responses of the different clones to MSeA after 16 h exposure. 100× magnification. (**C**) Induction of apoptosis by MSeA in the transfectant clones detected by death ELISA (24 h exposure). Each point represents mean and SD of four replicates. These data are representative of at least two independent experiments.

inhibitor-derived AKT inhibitor NL-71-101 (35) (Figure 5A) and with its specific 3-(hydroxymethyl)-bearing phosphatidylinositol ether lipid analog inhibitor (36) (Figure 5B) together made a compelling case. Furthermore, overexpression of a CA-AKT rendered DU145 cells more resistant to MSeA in direct proportion to the AKT activity of the transfectant clones (Figure 6). Therefore, irrespective of the androgen dependence status or p53 function (wild type in LNCaP, mutant in DU145), AKT activity can regulate apoptosis induced by MSeA in these cells. In further support of the assertion on p53 independence for MSeA-induced death, p53 Ser15 phosphorylation was not changed during the enhanced apoptosis execution by inhibitors of either AKT or ERK pathway (Figure 3B).

A second mitigating factor is the unexpected induction of ERK1/2 activation by MSeA, possibly an effect unique to the LNCaP cells because MSeA exposure decreases ERK1/2 phosphorylation in DU145 cells (14–16). Blocking ERK1/2 activation with U0126 slightly enhanced MSeA induction of apoptosis and resulted in a super-synergy when combined with the PI3K inhibitor (Figure 3). ERK1/2 activation therefore appears to be a compensatory survival response that offsets the apoptotic signaling induced by MSeA in LNCaP cells. The reasons for the two cell lines to show opposite patterns of ERK1/2 response to MSeA are not clear and require further

investigation. In contrast to the significant regulatory effects of AKT and ERK on MSeA-induced apoptosis in LNCaP cells, these kinases exerted little influence on apoptosis induced by selenite (Figure 2). These findings support the involvement of different protein kinase pathways for regulating apoptosis induction by different forms of selenium in PCa and other cell types (16). Such differences were also reflected in the caspase cascades activated as discussed next.

Our data showed that the general caspase inhibitor zVADfmk completely blocked apoptosis induced by MSeA in combination with PI3K and/or ERK inhibitor (Figure 4A), suggesting that AKT and ERK1/2 conferred resistance to MSeA-induced apoptosis ultimately through suppressing caspase activation pathways in LNCaP cells. In this scenario, when the suppression was relieved by one or both kinase inhibitors, MSeA-initiated signaling to caspase-8, caspase-9 and caspase-3 went through in variable degrees depending on which barrier was removed. The caspase cleavage patterns and activity assay results (Figure 3B and C) support a greater inhibition by AKT of mitochondria/cytochrome *c*/caspase-9 cascade than by ERK1/2 even though both kinase pathways attenuated caspase-8 activity with similar potency. The results of specific caspase inhibitors (Figure 4B) demonstrated that blocking caspase-8 pathway exerted a much greater protective effect against apoptosis induction than blocking caspase-9 pathway. This pattern of caspase activation by MSeA in LNCaP cells is essentially identical to that induced by MSeA in DU145 cells, wherein caspase-8 is a major initiator caspase upstream of caspase-9 (14). In contrast to these patterns, selenite-induced caspase-mediated apoptosis in LNCaP cells equally involved these two caspase cascades and was causally linked to p53 Ser15 phosphorylation, as we reported previously (34).

Our data suggest that AKT and ERK1/2 probably target different molecules in the extrinsic (major) and intrinsic (likely subordinate to caspase-8) caspase activation cascades to inhibit apoptosis induced by MSeA in LNCaP cells. Potential targets of AKT- and/or ERK1/2-mediated suppression of caspase pathways include an upregulation of the caspase-8 inhibitory proteins FLIPs by AKT (37,38) and ERK (39), AKT-mediated phosphorylation of BAD at serine136 (40,41), ERK-mediated phosphorylation of BAD at serine112 (42), AKT-mediated phosphorylative inactivation of caspase-9 (43), AKT-mediated phosphorylative inhibition of a Forkhead transcription factor (44) and AKT-induced expression of caspase-3 inhibitor protein survivin (45–47), to name a few. Some of these targets directly affect the activity of multiple caspases, and others impact mitochondria integrity and the intrinsic cascades. AKT has a number of targets affecting the intrinsic pathway and therefore was not surprising that we observed greatly enhanced mitochondrial release of cytochrome *c* by PI3K/AKT inhibition while MEK/ERK inhibition had no effect (Figure 3B). The actual target molecules for AKT and ERK pathways to regulate MSeA-induced apoptosis in PCa cells are being investigated.

In summary, the AKT activity played a critical role in regulating the sensitivity of LNCaP and DU145 PCa cells to the induction of apoptosis by MSeA. The MSeA-induced activation of ERK1/2 constituted an additional and possibly unique survival response that further rendered LNCaP cells less sensitive to MSeA. These kinase pathways conferred resistance by ultimately suppressing caspases, including both caspase-8 (major) and caspase-9 (minor) cascades, independent of p53. If applicable *in vivo*, our findings suggest that PCa cells with deregulated PTEN may be less susceptible to apoptosis by methylselenol, and combination with other agents that inhibit the PI3K-AKT signaling may improve overall chemoprevention efficacy. It is also possible that in a therapeutic context, targeted therapy through a combined use of methylselenium and PI3K/AKT inhibitors may improve the therapeutic outcome based on a prior knowledge of the PTEN–AKT profile of the tumors to be treated.

Acknowledgements

The authors thank Dr Zigang Dong for generously providing the AKT plasmid and Dr Howard Ganther for supplying MSeA. Supported in parts by Hormel Foundation, Eagles' Telethon, by grants from the National Cancer Institute (CA92231 and CA95642) and Department of Defense Prostate Cancer Research Program (DAMD17-02-1-0007).

Conflict of Interest Statement: None declared.

References

1. American Cancer Society. (2004) Cancer Facts & Figures.
2. Klein,E.A. and Thompson,I.M. (2004) Update on chemoprevention of prostate cancer. *Curr. Opin. Urol.*, **14**, 143–149.
3. Clark,L.C., Combs,G.F.,Jr, Turnbull,B.W. *et al.* (1996) Effects of selenium supplementation for cancer prevention in patients with carcinoma of the skin. A randomized controlled trial. Nutritional Prevention of Cancer Study Group. *JAMA*, **276**, 1957–1963.
4. Duffield-Lillico,A.J., Dalkin,B.L., Reid,M.E., Turnbull,B.W., Slate,E.H., Jacobs.E.T., Marshall,J.R. and Clark,L.C. (2003) Selenium supplementation, baseline plasma selenium status and incidence of prostate cancer: an analysis of the complete treatment period of the Nutritional Prevention of Cancer Trial. *Br. J. Urol.*, **91**, 608–612.
5. Klein,E.A., Thompson,I.M., Lippman,S.M., Goodman,P.J., Albanes,D., Taylor,P.R. and Coltman,C. (2001) SELECT: the next prostate cancer prevention trial. Selenium and Vitamin E Cancer Prevention Trial. *J. Urol.*, **166**, 1311–1315.
6. Stratton,M.S., Reid,M.E., Schwartzberg,G., Minter,F.E., Monroe,B.K., Alberts,D.S., Marshall,J.R. and Ahmann,F.R. (2003) Selenium and prevention of prostate cancer in high-risk men: the Negative Biopsy Study. *Anticancer Drugs*, **14**, 589–594.
7. Stratton,M.S., Reid,M.E., Schwartzberg,G., Minter,F.E., Monroe,B.K., Alberts,D.S., Marshall,J.R. and Ahmann,F.R. (2003) Selenium and inhibition of disease progression in men diagnosed with prostate carcinoma: study design and baseline characteristics of the 'Watchful Waiting' Study. *Anticancer Drugs*, **14**, 595–600.
8. Sinha,R. and El-Bayoumy,K. (2004) Apoptosis is a critical cellular event in cancer chemoprevention and chemotherapy by selenium compounds. *Curr. Cancer Drug Targets*, **4**, 13–28.
9. Ip,C. and Ganther,H.E. (1990) Activity of methylated forms of selenium in cancer prevention. *Cancer Res.*, **50**, 1206–1211.
10. Ip,C., Hayes,C., Budnick,R.M. and Ganther,H.E. (1991) Chemical form of selenium, critical metabolites, and cancer prevention. *Cancer Res.*, **51**, 595–600.
11. Ip,C., Thompson,H.J., Zhu,Z. and Ganther,H.E. (2000) *In vitro* and *in vivo* studies of methylseleninic acid: evidence that a monomethylated selenium metabolite is critical for cancer chemoprevention. *Cancer Res.*, **60**, 2882–2886.
12. Jiang,C., Ganther,H.E. and Lü,J. (2000) Monomethyl selenium-specific inhibition of MMP-2 and VEGF expression: implications for angiogenic switch regulation. *Mol. Carcinog.*, **29**, 236–250.
13. Lü,J. and Jiang,C. (2001) Antiangiogenic activity of selenium in cancer chemoprevention: metabolite-specific effects. *Nutr. Cancer*, **40**, 64–73.
14. Jiang,C., Wang,Z., Ganther,H.E. and Lü,J. (2001) Caspases as key executors of methyl selenium induced apoptosis (anoikis) of DU145 human prostate cancer cells. *Cancer Res.*, **61**, 3062–3070.
15. Wang,Z., Jiang,C. and Lü,J. (2002) Induction of caspase-mediated apoptosis and cell-cycle G_1 arrest by selenium metabolite methylselenol. *Mol. Carcinog.*, **34**, 113–120.

16. Jiang,C., Wang,Z., Ganther,H.E. and Lü,J. (2002) Distinct effects of methylseleninic acid versus selenite on apoptosis, cell cycle, and protein kinase pathways in DU145 human prostate cancer cells. *Mol. Cancer Ther.*, **1**, 1059–1066.
17. Lawlor,M.A. and Alessi,D.R. (2001) PKB/Akt: a key mediator of cell proliferation, survival and insulin responses. *Cell Sci.*, **114**, 2903–2910.
18. Nicholson,K.M. and Anderson,N.G. (2002) The protein kinase B/Akt signalling pathway in human malignancy. *Cell. Signal.*, **14**, 381–395.
19. Shiojima,I. and Walsh,K. (2002) Role of Akt signaling in vascular homeostasis and angiogenesis. *Circ. Res.*, **90**, 1243–1250.
20. Cairns,P., Okami,K., Halachmi,S., Halachmi,N., Esteller,M., Herman,J.G., Jen,J., Isaacs,W.B., Bova,G.S. and Sidransky,D. (1997) Frequent inactivation of PTEN/MMAC1 in primary prostate cancer. *Cancer Res.*, **57**, 4997–5000.
21. Pesche,S., Latil,A., Muzeau,F., Cussenot,O., Fournier,G., Longy,M., Eng,C. and Lidereau,R. (1998) PTEN/MMAC1/TEP1 involvement in primary prostate cancers. *Oncogene*, **16**, 2879–2883.
22. Feilotter,H.E., Nagai,M.A., Boag,A.H., Eng,C. and Mulligan,L.M. (1998) Analysis of PTEN and the 10q23 region in primary prostate carcinomas. *Oncogene*, **16**, 1743–1748.
23. Cantley,L.C. and Neel,B.G. (1999) New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase/AKT pathway. *Proc. Natl Acad. Sci. USA*, **96**, 4240–4245.
24. Johnson,G.L. and Lapadat,R. (2002) Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. *Science*, **298**, 1911–1912.
25. Chang,F., Steelman,L.S., Shelton,J.G., Lee,J.T., Navolanic,P.M., Blalock,W.L., Franklin,R. and McCubrey,J.A. (2003) Regulation of cell cycle progression and apoptosis by the Ras/Raf/MEK/ERK pathway. *Int. J. Oncol.*, **22**, 469–480.
26. Putz,T., Culig,Z., Eder,I.E., Nessler-Menardi,C., Bartsch,G., Grunicke,H., Uberall,F. and Klocker,H. (1999) Epidermal growth factor (EGF) receptor blockade inhibits the action of EGF, insulin-like growth factor I, and a protein kinase A activator on the mitogen-activated protein kinase pathway in prostate cancer cell lines. *Cancer Res.*, **59**, 227–233.
27. Uzgare,A.R. and Isaacs,J.T. (2004) Enhanced redundancy in Akt and mitogen-activated protein kinase-induced survival of malignant versus normal prostate epithelial cells. *Cancer Res.*, **64**, 6190–6199.
28. Lü,J., Kaeck,M., Jiang,C., Wilson,A.C. and Thompson,H.J. (1994) Selenite induction of DNA strand breaks and apoptosis in mouse leukemic L1210 cells. *Biochem. Pharmacol.*, **47**, 1531–1535.
29. Lü,J., Jiang,C., Kaeck,M., Ganther,H., Vadhanavikit,S., Ip,C. and Thompson,H. (1995) Dissociation of the genotoxic and growth inhibitory effects of selenium. *Biochem. Pharmacol.*, **50**, 213–9.
30. Zhou,N., Xiao,H., Li,T.K., Nur-E-Kamal,A. and Liu,L.F. (2003) DNA damage-mediated apoptosis induced by selenium compounds. *J. Biol. Chem.*, **278**, 29532–29537.
31. Earnshaw,W.C., Martins,L.M. and Kaufmann,S.H. (1999) Mammalian caspases: structure, activation, substrates, and functions during apoptosis. *Annu. Rev. Biochem.*, **68**, 383–424.
32. Wolf,B.B. and Green,D.R. (1999) Suicidal tendencies: apoptotic cell death by caspase family proteinases. *J. Biol. Chem.*, **274**, 20049–20052.
33. Duriez,P.J. and Shah,G.M. (1997) Cleavage of poly(ADP-ribose) polymerase: a sensitive parameter to study cell death. *Biochem. Cell Biol.*, **75**, 337–349.
34. Jiang,C., Hu,H., Malewicz,B., Wang,Z. and Lü,J. (2004) Selenite-induced p53 Ser-15 phosphorylation and caspase-mediated apoptosis in LNCaP human prostate cancer cells. *Mol. Cancer Ther.*, **3**, 877–884.
35. Reuveni,H., Livnah,N., Geiger,T., Klein,S., Ohne,O., Cohen,I., Benhar,M., Gellerman,G. and Levitzki,A. (2002) Toward a PKB inhibitor: modification of a selective PKA inhibitor by rational design. *Biochemistry*, **41**, 10304–10314.
36. Hu,Y., Qiao,L., Wang,S., Rong,S.B., Meuillet,E.J., Berggren,M., Gallegos,A., Powis,G. and Kozikowski,A.P. (2000) 3-(Hydroxymethyl)-bearing phosphatidylinositol ether lipid analogues and carbonate surrogates block PI3-K, Akt, and cancer cell growth. *J. Med. Chem.*, **43**, 3045–3051.
37. Panka,D.J., Mano,T., Suhara,T., Walsh,K. and Mier,J.W. (2001) Phosphatidylinositol 3-kinase/Akt activity regulates c-FLIP expression in tumor cells. *J. Biol. Chem.*, **276**, 6893–6896.
38. Suhara,T., Mano,T., Oliveira,B.E. and Walsh,K. (2001) Phosphatidylinositol 3-kinase/Akt signaling controls endothelial cell sensitivity to Fas-mediated apoptosis via regulation of FLICE-inhibitory protein (FLIP). *Circ. Res.*, **89**, 13–19.
39. Aoudjit,F. and Vuori,K. (2001) Matrix attachment regulates Fas-induced apoptosis in endothelial cells: a role for c-flip and implications for anoikis. *J. Cell Biol.*, **152**, 633–43.
40. del Peso,L., Gonzalez-Garcia,M., Page,C., Herrera,R. and Nunez,G. (1997) Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt. *Science*, **278**, 687–689.
41. Datta,S.R., Dudek,H., Tao,X., Masters,S., Fu,H., Gotoh,Y. and Greenberg,M.E. (1997) Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell*, **91**, 231–241.
42. Scheid,M.P., Schubert,K.M. and Duronio,V. (1999) Regulation of bad phosphorylation and association with Bcl-x(L) by the MAPK/Erk kinase. *J. Biol. Chem.*, **274**, 31108–31113.
43. Cardone,M.H., Roy,N., Stennicke,H.R., Salvesen,G.S., Franke,T.F., Stanbridge,E., Frisch,S. and Reed,J.C. (1998) Regulation of cell death protease caspase-9 by phosphorylation. *Science*, **282**, 1318–1321.
44. Brunet,A., Bonni,A., Zigmond,M.J., Lin,M.Z., Juo,P., Hu,L.S., Anderson,M.J., Arden,K.C., Blenis,J. and Greenberg,M.E. (1999) Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell*, **96**, 857–868.
45. Papapetropoulos,A., Fulton,D., Mahboubi,K., Kalb,R.G., O'Connor,D.S., Li,F., Altieri,D.C. and Sessa,W.C. (2000) Angiopoietin-1 inhibits endothelial cell apoptosis via the Akt/survivin pathway. *J. Biol. Chem.*, **275**, 9102–9105.
46. Tran,J., Rak,J., Sheehan,C., Saibil,S.D., LaCasse,E., Korneluk,R.G. and Kerbel,R.S. (1999) Marked induction of the IAP family antiapoptotic proteins survivin and XIAP by VEGF in vascular endothelial cells. *Biochem. Biophys. Res. Commun.*, **264**, 781–788.
47. Fornaro,M., Plescia,J., Chheang,S., Tallini,G., Zhu,Y.M., King,M., Altieri,D.C. and Languino,L.R. (2003) Fibronectin protects prostate cancer cells from tumor necrosis factor-alpha-induced apoptosis via the AKT/survivin pathway. *J. Biol. Chem.*, **278**, 50402–50411.

Received December 21, 2004; revised March 30, 2005; accepted April 10, 2005